Hydrolysis of Cassava Starch by Co-immobilized Multi-microorganisms of Loog-Pang (Thai rice cake starter) for Ethanol Fermentation

Arnon Khamkeaw and Muenduen Phisalaphong*

Chemical Engineering Research Unit for Value Adding of Bioresources, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand

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*Corresponding Author
Tel: +662-218-6875
Fax: +662-218-6877
E-mail: muenduen.p@chula.ac.th

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Abstract Loog-Pang (Thai rice cake starter) is an effective and inexpensive microbial source for the hydrolysis of cassava starch to glucose. A process for hydrolysis of cassava starch to glucose by Loog-Pang was improved by co-immobilized multi-microorganisms (IC) using thin shell silk cocoon (TSC). After incubation at 35°C for 120 h, the IC-TSC system converted 20% w/v cassava starch slurry into clear glucose syrup containing a glucose concentration of 145.5 g/L (composed of 98.8% glucose and 1.2% oligosaccharides), with little or no contamination by microorganisms. The glucose concentration from the starch hydrolysis process using the IC-TSC system was approximately 1.3 times more than that of suspended cultures (SC). The starch hydrolysate could be used as the carbon source for ethanol fermentation without sterilization. A concentration of ethanol of 71.2 g/L (9.1%, v/v) was obtained at 36 h fermentation of the starch hydrolysate by *Saccharomyces cerevisiae* M30.

Keywords: cassava starch, hydrolysis, immobilization, Loog-Pang, ethanol

Introduction

Cassava starch is one of the major raw materials used to produce sugar syrup because of its low cost and availability. Starch can be almost completely hydrolyzed to produce glucose syrup, which is further used for food, beverage and pharmaceutical industries (1). Glucose syrup from enzymatic hydrolysis of cassava starch or flour is also extensively used for ethanol fermentation for industrial and fuel markets (1,2). Cassava is easy to grow in tropical and subtropical areas such as Nigeria, Thailand, Brazil, and Indonesia, and its production is continuing to increase in Africa and Asia (3). It can be grown in a wide range of soils, from sands to clays and also in soils of low fertility where other agricultural materials, such as sugarcane, rice, and corn, cannot be grown well (4).

During the starch hydrolysis, starch is decomposed into glucose or to oligosaccharides by acid hydrolysis and/or enzymatic hydrolysis. Compared to acid hydrolysis, enzymatic hydrolysis occurs at milder condition and is more environmentally acceptable; therefore, acid hydrolysis of starch is now largely replaced by enzymatic process, in which glucose syrups can be produced in a two-step enzymatic process: liquefaction and saccharification. In the liquefaction stage, α -amylase randomly breaks α -1,4 linkages of starch to yield dextrin, maltose, maltotriose, and maltopentose. Then, in the saccharification stage, amyloglucosidase (or glucoamylase) break α -1,4 and α -1,6 glucosidic

linkages from the non-reducing end, yielding glucose (5). Amylolytic enzymes can be produced by many strains of yeasts, molds, and bacteria. Among these, *Rhizopus* spp. and *Aspergillus* spp. are considered good producers for industrial production of amylolytic enzyme (6,7). However, the high cost of enzymes significantly increases the production cost of glucose syrup (8). Rice cake starter is a traditional starter for starch hydrolysis to produce alcoholic foods used in many Asian countries. In Thailand, the rice cake starter is commonly called “Loog-Pang-Kao-Mhark” or “Loog-Pang”. In other Asian countries, rice cake starters have different local names such as “Banh-Men” in Vietnam, “Chu” or “Chinese yeast cake” in China, “Tan-koji” in Japan, “Nuruk” in Korea (9), “Marcha or Murchan” in India, “Ragi” in Indonesia, “Ragi tapai” in Malaysia, and “Bubod” in the Philippines (10). Loog-Pang is used for the production of alcoholic foods in Thailand, such as Kao-Mag (alcohol sweetened rice), Lao (rice wine), Nam Som Saichu (vinegar), and Kanomtuay foo (fully cup or Thai dry cake) (8). Loog-Pang comprises of multi-microorganisms of yeasts, molds, and bacteria, which have the ability to produce α -amylase and glucoamylase to convert starch to glucose and oligosaccharides and produce ethanol and some organic acids. Taking advantage of its low cost and availability, Loog-Pang is an effective and inexpensive microbial source for starch hydrolysis.

Immobilized bacteria or yeasts have been applied to batch and continuous ethanol fermentations owing to the feasibility for

repeated use with high cell density (11-15). A high density of immobilized cells (IC) can help improve the product yield and the volumetric productivity of bioreactors. Recently, our group has reported significant improvements in ethanol production from cane molasses using immobilized *Saccharomyces cerevisiae* M30 on thin-shell silk cocoons (TSC) due to its high mechanical strength, light weight, biocompatibility, high surface area, and cell protection (11). In this work, IC culture of Loog-Pang on TSC (IC-TSC) was used to improve the process for hydrolysis of cassava starch for sugar syrup production. The use of sugar syrup from the starch hydrolysis for ethanol fermentation was also investigated. The performance of the IC-TSC system in starch hydrolysis for sugar syrup production and the further use of the starch hydrolysate for ethanol fermentation were compared to those from the suspended culture (SC) system.

Materials and Methods

Microorganisms, culture media, and cell preparation Loog-Pang purchased from a local market (Phichit, Thailand), was used as a mixed culture for the cassava starch hydrolysis process. It was stored in sealed containers at 4°C. Loog-Pang was ground into a fine powder before being used as a powdered starter culture. *S. cerevisiae* M30 provided by the laboratory of Dr. Savitree Limtong (Department of Microbiology, Kasetsart University, Bangkok, Thailand) was used as a yeast culture for ethanol fermentation because of its high efficiency in ethanol production (13-15). Stock cultures were stored in a PDA agar slant at 4°C. A yeast starter was prepared by aseptically transferring a loop of the stock culture into a 500 mL Erlenmeyer flask containing 150 mL sterilized cultivation medium. The medium was composed of 100 g/L reducing sugars from cane molasses, 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/L KH_2PO_4 , and 0.035 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 5 (13-15). Cell cultivation was performed in an Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and 33°C for 24 h.

Immobilization support TSCs of mulberry silkworms were purchased from the Queen Sirikit Department of Sericulture (Saraburi, Thailand). TSC was used as a natural support material for cells in the process of cassava starch hydrolysis. TSCs in 500 mL flasks were autoclaved separately for 15 min at 121°C. The 2.5 g sterilized TSCs were immersed into 250 mL of 20% (w/v) starch slurry in 500 mL Erlenmeyer flasks prior to adding the powdered starter culture of Loog-Pang to induce natural cell adsorption on TSCs.

Cassava starch hydrolysis The slurry of cassava starch (20%, w/v) was prepared by mixing 50 g of cassava starch (ETC International Trading Co., Ltd., Bangkok, Thailand) with 250 mL distilled water in a 500 mL Erlenmeyer flask. It was gelatinized by cooking at 75°C for 1 h with gentle agitation (12). The gelatinized starch and the powdered starter culture (5%, w/w) were mixed in a 500 mL Erlenmeyer flask

and incubated in a refrigerated incubator shaker (Innova 4330; New Brunswick Scientific) at 150 rpm and 35 and 50°C for 144 h. Samples of 3 mL were collected every 12 h for sugar analysis. The samples of cassava starch hydrolysate were frozen before the analysis of the sugar concentrations to allow all samples to be analyzed at the same time. For the starch hydrolysis with the immobilized cells on TSC (IC-TSC), 2.5 g sterilized TSCs were added into 500 mL Erlenmeyer flasks containing 250 mL gelatinized starch slurry prior to adding the powdered starter culture of Loog-Pang. Then, the experiment was conducted and monitored in the same way as described for the system using the SC. All experiments were performed in duplicate.

Ethanol fermentation Batch fermentation experiments were performed in duplicate with the sugar syrup from cassava starch hydrolysis as the sole carbon source for *S. cerevisiae* M30. The medium was prepared by adding 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/L KH_2PO_4 , and 0.035 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and the initial pH was adjusted to 5.0. Experiments were performed in 500 mL Erlenmeyer flasks, with 250 mL total liquid volume. Experiments were initiated by transferring 5% of the starter culture to the prepared medium. Fermentation flasks were then shaken in an incubator at 150 rpm and 33°C for 72 h. The experiment was monitored by collecting samples of 5 mL every 12 h for sugar and ethanol concentration analysis. All experiments were performed in duplicate.

Analytical methods To measure the total sugar concentration, a 3 mL sample of the cassava starch hydrolysate was centrifuged at 755xg for 10 min. The glucose content in the samples was directly determined by a YSI (Model 2700) SELECT Bio-chemistry Analyzer from YSI Incorporated (Yellow Springs, OH, USA). To analyze the content of oligosaccharides, the sample solution was hydrolyzed with 370 g/L HCl in a boiling water bath for 10 min. After hydrolysis, the sample was neutralized by 200 g/L NaOH (13-15). The total reducing sugar content from the hydrolyzed sample was then determined using the 3,5-dinitrosalicylic acid (DNS) method (16). The oligosaccharide contents were determined from the differences between the total reducing sugar contents in the samples after the hydrolysis and the glucose contents in the samples before the hydrolysis.

Amylase activity was assayed according to Bernfeld's method (17). The reaction mixture containing 0.5 mL of 1% soluble starch solution dissolved in 0.02 M sodium phosphate buffer of pH 6.9 with 0.006 M sodium chloride and 0.5 mL of enzyme solution were incubated at 35°C for 5 min. After that, the reaction was stopped by adding 1.0 mL of DNS reagents and the samples were placed in boiling water bath for 5 min and cooled in ice bath. Then, 10 mL of distilled water was added into each sample and the mixture was thoroughly mixed on a vortex mixer. Afterward, the absorbance of each solution at 520 nm was measured by UV-Visible spectro-photometer (UV-2450; Shimadzu, Kyoto, Japan). A unit of enzyme activity was defined as the amount of enzyme that produces 1.0 µg of glucose as reducing sugar per

minute under the assay conditions.

The concentrations of ethanol were determined by a gas chromatography system using a Shimadzu Model GC-7AG equipped with a flame ionization detector (GC-7AG; Shimadzu). A column (0.125 cm i.d., 2 m, stainless steel (SS) packed with Porapak Q 80-100 mesh (Bara Scientific Co., Ltd., Bangkok, Thailand) was used with N₂ as the carrier gas at the flow rate of 50 mL/min. The injector temperature was 280°C, and the detector temperature was 300°C (13-15).

To provide a visual characterization of the cells and support material, a series of Scanning Electron Microscopy (SEM) images was taken. The samples were frozen in liquid nitrogen, immediately snapped, vacuum-dried, sputtered with gold and then photographed. Images were collected on a JEOL (Tokyo, Japan) JSM-5410LV scanning electron microscope.

Results and Discussion

Cassava starch hydrolysis by suspended cultures (SC) of Loog-Pang

Most studies have found *Saccharomycopsis fibuligera* as the common yeast in Loog-Pang (8,18,19). The optimal temperature and pH for glucoamylase and α -amylase of *S. fibuligera* were 40-50°C and 5.0-6.2, respectively (19) and it was shown that the increase of the inoculum size of *S. fibuligera* from 3 to 10% did not significantly affect the reducing sugar production (8). The results of our preliminary study of optimal conditions for cassava starch hydrolysis using the SC of Loog-Pang agree well with those of previous reports (8,18,19). It was found that the optimal conditions for the starch hydrolysis were 35-50°C, initial pH 5.0 and 5% inoculum (w/w of dry cassava starch) and the optimal concentration of starch slurry was 20% (w/v) (data not shown). The sugar concentration profiles from the hydrolysis at 20% (w/v) cassava starch using the powdered Loog-Pang in the forms of SC at the optimal conditions are shown in Fig. 1A and 1C. Rapid hydrolysis of the starch occurred in the first 12 h, during which the oligosaccharide and glucose concentrations continuously increased. After 12 h, the concentration of oligosaccharides was gradually decreased because they were broken into glucose by the amylolytic enzymes from microorganisms in the Loog-Pang. At 132 h of hydrolysis under the operating temperature of 35 and 50°C, the glucose concentrations in the hydrolysates were 114.4 and 111.7 g/L and oligosaccharide concentrations were 5.4 and 69.9 g/L, respectively. The hydrolysate obtained from the system using the SC of Loog-Pang was a cloudy white suspension as shown on the bottom left of Fig. 1. The higher oligosaccharide concentration was obtained by starch hydrolysis at 50°C as compared to that at 35°C, whereas slightly higher glucose concentration was obtained at 35°C. The activities of amylolytic enzymes from the hydrolysates of the systems using Loog-Pang in forms of SC are shown in Fig. 2. The amylase activity from the starch hydrolysate at 50°C was relatively higher than that at 35°C during 0-12 h. The amylase activity was highest at 12 h of the starch

hydrolysis and gradually decreased after that.

Amylase is an exoenzyme, which is secreted by cells and hydrolyzes starch into mono- and disaccharide subunits (20). Generally, chemical reactions increase with rise in temperature; however, if the temperature is too high, the amylolytic enzymes (α -amylase and glucoamylase) can be denatured. From our examination, no (or very low) starch hydrolysis was observed at cultivation temperatures of 60°C or above. The optimal temperature allowing the maximal catalytic activity by *Schwanniomyces castellii* was 45°C for α -amylase and 45-52°C for glucoamylase and a rapid decrease in both activities was observed just above those temperatures (20). From previous reports (21,22), the optimal temperature for amylase production by molds was 30-35°C. For the hydrolysis of cassava starch by microorganisms in Loog-Pang, the operating conditions should be suitable for amylase production and for hydrolytic activity of the amylase enzymes. According to the report on the saccharification of cassava starch by *S. fibuligera* isolated from Loog-Pang, the maximum reducing sugar at 46 g/L was obtained after 120 h (8). It was demonstrated in this study that a significant higher reducing sugar concentration (greater 100 g/L) could be obtained by using co-hydrolysis of the multi-microorganisms of Loog-Pang.

Cassava starch hydrolysis by co-immobilized multi-microorganisms of Loog-Pang on TSC (IC-TSC)

As mentioned previously, the optimal temperature for hydrolysis of cassava starch by the starter of Loog-Pang in the form of SC was 35-50°C. However, it was found that the optimal temperature for starch hydrolysis using the co-immobilized multi-microorganisms of Loog-Pang on TSC (IC-TSC) was 35°C (Fig. 1B). A slight adsorption of cells on TSC was observed at 38°C (data not shown). The operating temperature at 38°C or above can inhibit growth and proliferation of many microorganisms and might also destroy some microbial cells. A considerable drop of amylase production was observed at the operating temperature 40°C (21,22).

In Thailand, Loog-Pang is made from rice flour mixed with a variety of herbs and spices such as galanga, garlic, long pepper, licorice, and black pepper, including a powdered starter from a previous batch (23). It was revealed that a microbial community in Loog-pang had been dominated by molds (*Amylomyces* sp., *Mucor* sp. and *Rhizopus* sp.) and yeasts (*S. fibuligera*) at cell densities of around 3.8×10^6 and 5.1×10^6 CFU/g, respectively (23). Similar results from the identification of microorganisms in Loog-pang (Thailand) were previously reported (10,18). Generally, Loog-Pang is used for alcoholic food production at the ambient temperature in Thailand, where the average daytime temperature is usually between 30-36°C (18). The sugar concentration profiles obtained from the system using the IC-TSC system at 35 and 50°C are shown in Fig. 1B and 1D. During starch hydrolysis, the glucose concentration increased with longer incubation times. Meanwhile, the concentration of oligosaccharides increased in the first stage of the hydrolysis (12 h) and then gradually decreased. The maximum total concentration of reducing sugars and oligosaccharides was 154.8 g/L (at 72 h), whereas the maximum glucose concentration

Fig. 1. The top is the comparison of sugar concentration profiles (■: glucose; ---●---: oligosaccharides; ▲: total concentration) from the hydrolysis of cassava starch using Loog-Pang in forms of SC (A and C) and IC-TSC (B and D) at temperatures of 35°C (A and B) and 50°C (C and D). The bottom is the appearance of the hydrolysates from the hydrolysis of cassava starch for 144 h using Loog-Pang in forms of SC (left) and IC-TSC (right).

was 145.5 g/L (at 35°C, 120 h). After 120 h (35°C) in the IC-TSC system, almost all of the oligosaccharides were converted to glucose. The hydrolysate solution resembled sugar syrup, containing 98.8% glucose and 1.2% oligosaccharides (dry basis). Although the maximum total sugar and oligosaccharide content in the hydrolysate was lower than that of the SC system, the maximum glucose concentration obtained from the IC-TSC system was approximately 1.3 times that of the SC system. The highest activity of amylolytic enzymes was observed at 12 h of the starch hydrolysis at 35°C in the IC-TSC system (Fig. 2A), which agrees with the starch hydrolysis result in Fig. 1. It was also observed that some microbial strains in Loog-Pang could use sugars as a carbon source to produce products such as ethanol and some organic acids, which could be detected from the increase in ethanol concentration (to approximately 12.5 g/L) and the decrease in pH (from 5.0 to 2.8) at the end of the starch hydrolysis. As compared to the result from the use of Loog-Pang in the form of SC, it was demonstrated that the use of Loog-Pang in the form of IC-TSC could enhance glucose yield from the starch hydrolysis. The reduction of glucose feedback inhibitory effect in the IC-TSC system on production of amylases might be the reason for the improved

glucose productivity (24). Previously, the applications of immobilized yeast cells on TSC also showed the potential benefit for reduction of the inhibitory effect of sugars on ethanol productivity (13). It should be noted that for both SC and IC-TSC systems under the operating temperature of 35°C, almost oligosaccharides were converted into glucose after 132 h of the hydrolysis, whereas the hydrolysates from the starch hydrolysis at 50°C contained very high oligosaccharide concentration and less glucose concentration.

The quality of glucose syrup from the cassava hydrolysis was improved by the modified hydrolysis process using the IC-TSC system as shown in Fig. 1 (on the bottom right). Based on our observations of cells under a microscope, there was no contamination by microorganisms in the hydrolysate prepared from the system using IC-TSC (data not shown), which it means that there was no (or very low) release of the microbial cells from the immobilizing material (TSC containing cells). Because all (or almost all) of the microorganisms were attached onto the TSC surface, there was little or no contamination by microorganisms from Loog-Pang in the hydrolysate solution when using the IC-TSC. Therefore, the hydrolysate solution in the form of clear glucose syrup from the IC-TSC system could be

Fig. 2. Amylase activities from starch hydrolysates of the systems using Loog-Pang in forms of SC (○) and IC-TSC (●) at temperatures of 35°C (A) and 50°C (B).

used as a raw material for the ethanol fermentation process without sterilization. The overall appearance of TCS before and after the hydrolysis of cassava starch for 120 h is shown in Fig. 3A and 3B, respectively. SEM was used to visualize the microorganisms on TSC. An enlarged view of the TSC surface before the cell immobilization is shown in Fig. 3C. After the addition of 5% (w/w) powdered starter of Loog-Pang into 250 mL starch slurry containing 2.5 g sterilized TSCs and incubation at 35°C for 24 h, biofilms from natural adsorption of multi-microorganisms from Loog-Pang to TSC surfaces could be observed. The SEM image of the enlarged view of the TSC surface after colonization by the multi-microorganisms from the Loog-Pang for 120 h of the incubation is shown in Fig. 3D. The immobilized microorganisms attached to the TSC carriers can be divided into 3 groups: yeasts (Fig. 3E), molds (Fig. 3F), and bacteria (Fig. 3G), which are similar to those from previous investigations. According to the classification of the microorganisms in Loog-Pang from many previous works (8-10,18,25,26), *S. fibuligera* strains are common yeasts found in Loog-Pang (Thai) and other rice starter cakes. From the study of yeasts in Loog-Pang (Thai) and Loog-Pang (Lao), the most isolated yeasts were *S. fibuligera* and the minority ones included *Torulaspora globosa*, *Issatchenkia orientalis*, *C. glabrata*, *Pichia anomala*, and *P. burtonii* (8). *S. fibuligera* has been known to produce high amylolytic activity (8,10,18), whereas *T. globosa*, *I. orientalis*, *P. anomala*, and *P. burtonii* produce ethanol (8,10,18). *R. oryzae*, *R. microsporus*, *Absidia corymbifera*, *Amylomyces* sp., and *S. fibuligera* were members of the amylase producer group, and *S. cerevisiae*, *Issatchenkia* sp., *P. anomala* were members of the ethanol producer group (25). Overall, the microorganisms in Loog-Pang or rice starters are classified into two main groups. The first group is microorganisms that produce amylolytic enzymes for the digestion of starch into oligosaccharides and sugars; the majority ones are yeast strains of *S. fibuligera* and starch hydrolysis molds such as *Amylomyces* sp., *Absidia corymbifera*, *Rhizopus oryzae*, *R. microspores*. Then, sugars are converted to ethanol by yeasts (*S. cerevisiae*, *Issatchenkia* sp., *P. anomala*) and to organic acids by acid bacteria (*Acetobacter* sp., lactic acid bacteria) in

the second group (8,10,18,25,26).

In summary, the major advantages of the modified hydrolysis process using Loog-pang in the IC-TSC system are (1) enhanced glucose yield, (2) high purity glucose syrup with little or no contamination of microorganisms, and (3) they are energy saving because the hydrolysis temperature is low (35°C) and the hydrolysate can be used by yeasts for ethanol fermentation without sterilization. Moreover, the immobilization process allows for repeated use of the microorganisms. However, for the use of the IC-TSC system in repeated batch or continuous hydrolysis processes over a long period of time, some further improvements are required, such as the development of a culture medium to maintain cell activities and stability. To the best of our knowledge, the immobilization of multi-microorganisms of Loog-Pang on support materials (TSC) for starch hydrolysis similarly to our approach has not been previously reported. A method of cell immobilization by natural adsorption is simple, cheap and reusable. TSC is considered an excellent support material for natural cell adsorption due to its special microstructure with high porosity and high surface area. Additional advantages of TSC are its biocompatibility, light weight, reusability, high mechanical strength, and low cost. For some other methods, cell attachment on the carrier surface might have to be induced by linking agents, such as metal oxides or covalent-bonding agents, which may cause significant loss of cell activities (27).

Ethanol fermentation Ethanol fermentation by *S. cerevisiae* M30 was conducted in batch mode (Fig. 4). The hydrolysates obtained from the starch hydrolysis by the SC and IC-TSC systems were used as a carbon source. The hydrolysates were sterilized at 121°C for 15 min before use for ethanol fermentation. The ethanol concentrations from both systems were almost constant after 24-36 h of fermentation. The maximum ethanol concentration of 52.3 g/L (6.7%, v/v) was obtained after 48 h fermentation of the hydrolysate from the SC system (35°C). The maximal ethanol concentration at 71.2 g/L (9.1%, v/v) was obtained at 36 h fermentation of the hydrolysate from the

Fig. 3. The top is a thin shell silk cocoon (TSC) of mulberry silkworm: (A) initial appearance and (B) after 120 h of colonization by microorganism of Loog-Pang in the hydrolysis of cassava starch. The bottom is the enlarged view of TSC surface before (C) and after (D) colonization by microorganisms from Loog-Pang for 120 h; microbial cells from Loog-Pang on TSC could be divided into 3 groups: (E) yeast (arrow heads), (F) molds and (G) bacteria (arrow heads).

IC-TSC system (35°C). The ethanol conversion yield was 48.93% (approximately 96% of the theoretical yield). After 36 h of fermentation, the ethanol concentration slightly decreased. It was previously reported that when the concentration of fermentable sugars was very low, yeast cells might use ethanol as a carbon and energy source, resulting in a lower ethanol concentration (28).

Only glucose in both systems could be almost completely converted into ethanol, whereas oligosaccharides from the incomplete hydrolysis of the cassava starch were slightly used for ethanol production by *S. cerevisiae* M30. More complete hydrolysis of starch into glucose was obtained at the hydrolysis temperature of 35°C. In addition, the enhanced glucose production was obtained from the IC-TSC system, resulting in the highest ethanol production when using the hydrolysates from the IC-TSC system at 35°C (Fig. 4B).

From our examination, no significant difference in ethanol production was observed by the use of sterilized and non-sterilized hydrolysates from the IC-TSC system for ethanol fermentation (data not shown). On the other hand, the starch hydrolysate prepared from the SC system by using the powdered starter of Loog-Pang without immobilization contained lots of microorganisms from Loog-

Pang, making the hydrolysate appear white and cloudy (Fig. 1, on the bottom left). It was found that if non-sterilized hydrolysate from the SC system was used for ethanol fermentation, the final ethanol concentration would be reduced to only 20-25 g/L (data not shown), which was due to the inhibitory effect of the contaminating microorganism on the growth and ethanol fermentation activity of *S. cerevisiae*.

The difference in ethanol production and required fermentation time was mainly caused by the type and concentration of sugars in the hydrolysates. Yeasts can metabolize monosaccharides, such as glucose, fructose, mannose, and galactose, and disaccharides, such as maltose, sucrose, and lactose. Tetrasaccharides or other long chains of glucose units cannot be utilized by yeasts (29). Because glucose is a monosaccharide (a single sugar molecule), yeast can easily use glucose directly for ethanol production. For long-chain oligosaccharides, the bonds between the glucose subunits must be hydrolyzed to release monosaccharides or disaccharides before yeasts are able to convert them to ethanol. The concentration of sugars also affects the rate of fermentation. Generally, the ethanol concentration increased along with the increase in the initial sugar

Fig. 4. Concentrations of ethanol ($\triangle$), glucose ($\blacksquare$), and oligosaccharide ($\bullet$) during batch fermentations by *S. cerevisiae* M30 using the hydrolysates from the SC (A and C) and IC-TSC (B and D) systems at temperatures of 35°C (A and B) and 50°C (C and D).

concentration (30); however, very high initial sugar concentrations could induce substrate inhibition effects on the ethanol fermentation processes (13,15). Previously, for the alcohol produced from the cooked cassava starch slurry in simultaneous saccharification fermentation (SSF) by the co-culture of selected yeast strains from tan-koji and *S. cerevisiae*, 14.4 g/L of ethanol was obtained from 6% cassava starch medium after 24 h of fermentation (31).

In conclusion, Loog-Pang was an effective and inexpensive microbial source for starch hydrolysis. TSCs of mulberry silkworms were successfully used as a natural support material for the co-immobilization of multi-microorganisms of Loog-Pang in the process of cassava starch hydrolysis. After 120 h of hydrolysis of 20% (w/v) cassava starch slurry at 35°C using Loog-Pang in the IC-TSC system, almost all of the oligosaccharides were converted to glucose. The hydrolysate solution was in the form of clear glucose syrup, which was composed of 98.8% glucose and 1.2% oligosaccharides (dry basis). The maximum glucose concentration was 145.5 g/L, which was approximately 1.3 times of that of the system using the SC. The modified starch hydrolysis process by Loog-Pang in the IC-TSC system has many advantages, including the increased glucose yield and the purity of the glucose syrup. Because the reaction temperature was only 35°C and the hydrolysate could be further used by yeast cells for ethanol fermentation with no need to sterilize the hydrolysate; thus, the process required less energy. An ethanol concentration of 71.2 g/L (9.1%, v/v) was obtainable from the fermentation of the

hydrolysate for 36 h. The ethanol conversion yield was 48.9%, which was approximately 96% of the theoretical yield. Additionally, this process might be applied for the preparation of high-yield and high-purity glucose syrup from starch hydrolysis for other purposes.

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References

1. Chaisinboon O, Chontanawat J. Factors determining the competing use of Thailand's cassava for food and fuel. *Energy Procedia* 9: 216-229 (2011)
2. Moriizumi Y, Suksri P, Hondo H, Wake Y. Effect of biogas utilization and plant co-location on life-cycle greenhouse gas emissions of cassava ethanol production. *J. Clean. Prod.* 37: 326-334 (2012)
3. Shetty JK, Strohm BA, Lee SH, Duan G, Bates D. Cassava, the next corn for starch sweeteners. *Ind. Biotechnol.* 10: 275-284 (2014)
4. Nadir N, Mel M, Karim MIA, Yunus RM. Comparison of sweet sorghum and cassava for ethanol production by using *saccharomyces cerevisiae*. *J. Appl. Sci.* 9: 3068-3073 (2009)
5. Morales S, Álvarez H, Sánchez C. Dynamic models for the production of glucose syrups from cassava starch. *Food Bioprod. Process.* 86: 25-30 (2008)
6. Takahashi T, Kawauchi S, Suzuki K, Nakao E. Bindability and digestibility of high pressure-treated starch with glucoamylase from *Rhizopus* sp. *J. Biochem.* 116: 1251-1256 (1994)

7. Gomes E, Souza SR, Grandi RP, Silva RD. Production of thermostable glucoamylase by newly isolated *Aspergillus flavus* A 1.1 and *Thermomyces lanuginosus* A 13.37. *Braz. J. Microbiol.* 36: 75-82 (2005)
8. Saelim K, Dissara Y, Kittikun AH. Saccharification of cassava starch by *Saccharomycopsis fibuligera* YCY1 isolated from Loog-Pang (rice cake starter). *Songklanakarin J. Sci. Technol.* 30: 65-71 (2008)
9. Park JH, Chung CH. Characteristics of takju (a cloudy Korean rice wine) prepared with nuruk (a traditional Korean rice wine fermentation starter), and identification of lactic acid bacteria in nuruk. *Korean J. Food Sci. Technol.* 46: 153-164 (2014)
10. Limtong S, Sintara S, Suwanarit P, Lotong N. Species diversity of molds in Thai traditional fermentation starters (Loog-Pang). *Kasetsart J. (Nat. Sci.)* 39: 511-518 (2005)
11. Wirawan F, Cheng CL, Kao WC, Lee DJ, Chang JS. Cellulosic ethanol production performance with SSF and SHF processes using immobilized *Zymomonas mobilis*. *Appl. Energ.* 100: 19-26 (2012)
12. Amutha R, Gunasekaran P. Production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus*. *J. Biosci. Bioeng.* 92: 560-564 (2001)
13. Rattanapan A, Limtong S, Phisalaphong M. Ethanol production by repeated batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons. *Appl. Energ.* 88: 4400-4404 (2011)
14. Eiadpum A, Limtong S, Phisalaphong M. High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 114: 325-329 (2012)
15. Phisalaphong M, Budiraharjo R, Bangrak P, Mongkolkajit J, Limtong S. Alginate-loofa as carrier matrix for ethanol production. *J. Biosci. Bioeng.* 104: 214-217 (2007).
16. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428 (1959)
17. Bernfeld P. Amylase, alpha and beta. *Method Enzymol.* 1: 149-158 (1955)
18. Limtong S, Sintara S, Suwanarit P, Lotong N. Yeast diversity in Thai traditional alcoholic starter. *Kasetsart J. (Nat. Sci.)* 36: 149-158 (2002)
19. Hostinová E. Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera*. *Biologia* 11(Suppl.57): 247-251 (2002)
20. Clementi F, Rossi J. Alpha-amylase and glucoamylase production by *Schwanniomyces castellii*. *Anton. Leeuw. Int. J. G.* 52: 343-352 (1986)
21. Bedan DS, Aziz GM, Al -Sa'ady AJR. Optimum conditions for α -amylase production *Aspergillus niger* mutant isolate using solid state fermentation. *Curr. Res. Microbiol. Biotechnol.* 2: 450-456 (2014)
22. Swetha Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A. Alpha amylase production by *Aspergillus oryzae* employing solid-state fermentation. *J. Sci. Ind. Res.* 66: 621-626 (2007)
23. Chaijamrus S, Mouthung B. Selection of Thai starter components for ethanol production utilizing malted rice from waste paddy. *Songklanakarin J. Sci. Technol.* 33: 163-170 (2011)
24. Reddy CA, Abouzied MM. Glucose feedback inhibition of amylase activity in *Aspergillus* sp. and release of this inhibition when cocultured with *Saccharomyces cerevisiae*. *Enzyme Microb. Tech.* 8: 659-664 (1986)
25. Thanh VN, Mai LT, Tuan DA. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int. J. Food Microbiol.* 128: 268-273 (2008)
26. Tsuyoshi N, Fudou R, Yamanaka S, Kozaki M, Tamang N, Thapa S, Tamang JP. Identification of yeast strains isolated from marcha in sikkim, a microbial starter for amylolytic fermentation. *Int. J. Food Microbiol.* 99: 135-146 (2005)
27. Willaert RG, Baron GV. Gel entrapment and micro-encapsulation: Methods, applications and engineering principles. *Rev. Chem. Eng.* 12: 160-205 (1996)
28. Takeshige K, Ouchi K. Effects of yeast invertase on ethanol production in molasses. *J. Ferment. Bioeng.* 79: 513-515 (1995)
29. Yoon S, Mukerjee R, Robyt J. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydr. Res.* 338: 1127-1132 (2003)
30. Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H. Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. *Biomass Bioenerg.* 47: 395-401 (2012)
31. Amornpitak TY. Ethanol production from cassava starch by selected fungi from Tan-Koji and *Saccharomyces cerevisiae*. *Biotechnology* 9: 84-88 (2010)